Identification of Endogenous Gibberellins in Immature Navel Orange Fruit

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Eight gibberellins (GAs) were identified from the immature fruit of navel orange trees (*Citrus sinensis* L. Osbeck cv. Washington) after sequential purification by reverse-phase C_{18} high-performance liquid chromatography, Nucleosil $5N(CH_3)_2$ high-performance liquid chromatography, and capillary gas chromatography–mass spectrometry. GA_1, GA_4, GA_9, GA_{17}, GA_{19}, GA_{20}, and GA_{29} were identified on the basis of their full-scan mass spectra and Kovats retention indices. GA_8 was tentatively identified on the basis of the comparison of its full-scan mass spectrum with the published spectrum.

INTRODUCTION

Gibberellins (GAs) directly influence the yield, quality, and economic return of citrus fruits. The use of GA_3 on citrus is one of the major applications of GAs in agriculture. It is used mainly on navel and Valencia oranges to improve the rind quality of fruit for the fresh fruit market (Coggins and Henning, 1988; Martin, 1983). GAs seem to be involved in the regreening of Valencia oranges, which reduces their commercial value. Valencia oranges that have regreened show a marked increase in the GA-like activity, as measured by means of bioassay, before the chlorophyll begins to increase, while the GA-like activity in nonregreened fruit remained low (Rasmussen, 1973). The application of exogenous GA_3 in April increases the regreening observed in June and July (Rasmussen, 1973). Proper timing can minimize the effect of GA_3 on regreening in Valencia oranges but will still improve rind quality (Coggins and Henning, 1988). There has also been recent interest in the use of some of the newer gibberellin biosynthesis inhibitors to control the growth of citrus trees (Bausher and Yelenosky, 1987; Hadlow and Allan, 1989; Harty and Van Staden, 1988; Sakovich and Arpaia, 1987) to promote flower induction (Harty and Van Staden, 1988) and to induce cold hardiness (Harty and Van Staden, 1988; Mauk et al., 1987). The endogenous GAs of the fruit must be identified before quantitative measurements of individual GAs can be made and related to the control of regreening or other factors affecting fruit quality and yield.

Although GAs have important effects on citrus growth and fruit quality, until recently there have been only two papers published that attempted to identify the GAs in citrus. GA_1 was isolated from the water sprouts of mandarin orange (*Citrus unshiu*) and identified by infrared spectroscopy. This was one of the earliest identifications of a GA in higher plants (Kawarada and Sumiki, 1959). Washington navel oranges (*Citrus sinensis* L. Osbeck) and Eureka lemons (*Citrus limon* L. Burmann) were extracted to give three GA-like substances, two of which behaved similarly to GA_1 and GA_9 on the basis of cochromatography, fluorometric properties, and bioassays (Khalifah et al., 1965). More recently, we have used HPLC and GC–MS to identify GA_1, GA_{17}, GA_{19}, GA_{20}, GA_{29}, and iso-GA_3 and to tentatively identify GA_8 and GA_{44} in vegetative shoots of navel oranges (*C. sinensis* L. Osbeck cv. Washington) (Poling and Maier, 1988). Subsequently, GA_1, GA_3, GA_8, GA_{19}, GA_{20}, GA_{29}, 3-epi-GA_1, 2-epi-GA_{29}, and iso-GA_3 were identified in vegetative and reproductive tissues of Valencia oranges (*C. sinensis* L. Osbeck) (Turnbull, 1989), and GA_1, GA_4, GA_9, GA_{19}, GA_{20}, GA_{24}, GA_{25}, GA_{44}, and GA_{53} were identified in young fruit of Satsuma mandarin (*C. unshiu* Marc. cv Hayashi) (Goto et al., 1989). This paper extends our earlier work to include the identification of the endogenous gibberellins of immature navel orange fruit.

MATERIALS AND METHODS

Plant Material. Immature Washington navel oranges (*C. sinensis* L. Osbeck) were collected from a single small tree growing outdoors at our laboratory. The sample consisted of the fruit with the calyx and a short section of stem attached. The first year, the fruit were 3–10 mm in diameter, while the second year, they were 12–25 mm in diameter. The larger fruit were cut into pieces. Then the fruit were immediately frozen with dry ice, lyophilized, and stored at −14 °C prior to extraction. The dry weights for the first and second year were 74 and 103 g, respectively.

Extraction and Purification of the GAs from Navel Orange Fruit. Freeze-dried fruit from both years was extracted, in 88.5-g lots, as previously described (Poling and Maier, 1988) except for a few minor changes noted below. The sample was extracted with acetone/water (4:1 v/v) and filtered. The fruit extract was more difficult to filter than the shoot extract, so Celite 503 was added to the extract and a thin layer of Celite 503 was placed over the filter paper before filtration. The filtrates were treated with a mixture of activated charcoal and Celite, filtered, and reduced to an aqueous residue, which was partitioned against hexane after the addition of 0.5 M potassium phosphate buffer (pH 8.0). The aqueous extract was treated with polyvinylpolypyrrolidone (PVPP), filtered, and adjusted to pH 2.5 before being extracted with EtOAc. The EtOAc extract was washed, dried, and reduced to dryness to give an acidic EtOAc fraction of 1.47 g.

The acidic EtOAc fraction was separated by means of chromatography on a charcoal/Celite column and partitioned into EtOAc as previously described (Poling and Maier, 1988), except that the aqueous residue from the acetone/water (4:1 v/v) eluate was not treated with PVPP but was directly adjusted to pH 2.5 and extracted with EtOAc. This reduced the weight of the acidic EtOAc fraction to 150 mg.

The acidic EtOAc fraction was dissolved in 20 mL of MeOH, and 2-mL aliquots were processed through individual NH_2 Sep-Paks (Millipore, Milford MA) (Poling and Maier, 1988). The 1% (v/v) HOAc/MeOH fractions from all the aliquots were combined and, after 50 mL of toluene had been added to remove HOAc as an azeotrope, evaporated to dryness under reduced pressure at 35 °C. This reduced the dry weight of the GA fraction to 6.3 mg.

The GA fraction was separated by means of preparative C_{18} HPLC (Poling and Maier, 1988). Fractions from 6 to 18, from 18 to 24, and from 39 to 56 min were collected. Between 24 and

36 min, 3-min fractions were collected. The fractions from the C_{18} column were further separated by means of a Nucleosil 5N-$(CH_3)_2$ column (Macherey-Nagel, Duren, FRG) (Poling and Maier, 1988), except that the column was eluted with 0.05% (v/v) HOAc/MeOH at 0.9 mL/min. After all the peaks from the injection had been eluted, a standard solution of GA_3 was injected and the retention times (Rts) from the previous injection were divided by the Rt of GA_3 to give RRt(GA_3) (Rt relative to Rt of GA_3). For RRt(GA_3) from 0.5 to 1.5, three fractions of approximately equal volume were collected for each fraction from the C_{18} column. Each fraction was derivatized and analyzed by GC–MS as described below.

Reference Materials. GA_{20} was a gift from Dr. I. Railton. $MeGA_9$ was purchased from Sigma Chemical Co. (St. Louis, MO). GA_4 was separated from GA_7 (ICN Pharmaceuticals, Inc., Cleveland, OH) by means of a Nucleosil 5N$(CH_3)_2$ column using the same condition as for the navel orange fruit. Reference spectra and KRIs (Kovats retention index) for GA_{17}, GA_{19}, and GA_{29} were obtained as described before (Poling and Maier, 1988) after a 1-mL aliquot from the Progress 9 pea extract was separated by means of a Nucleosil 5N$(CH_3)_2$ column using the same condition as for the navel fruit.

Synthesis of $MeGA_1$ and GA_{20}. GA_3 (Sigma) was methylated with diazomethane and oxidized with pyridinium dichromate in dimethylformamide (Duri et al., 1981) or manganese dioxide in 1,4-dioxane (Beale and MacMillan, 1980). Stepwise elution with increasing amounts of EtOAc in hexane from a column packed with silica gel (Woelm Dry-Column, ICN Pharmaceuticals), followed by crystallization from EtOAc–hexane, gave the 3-enone of $MeGA_3$. This was dissolved in MeOH containing copper(I) chloride and reduced with sodium borohydride (Duri et al., 1981). $MeGA_1$ and Me-3-epi-GA_1 were separated by stepwise elution with EtOAc in hexane from a column packed with silica gel. These two fractions were further purified by means of HPLC using a Whatman Partisil M9 10/50 preparative column. The column was eluted at 9 mL/min with 2-propanol/CH_3CN/CH_2Cl_2/hexane (2:24:24:50 v/v) and monitored at 210 nm. Crystallization of the $MeGA_1$ fraction from the column, using EtOAc/hexane gave $MeGA_1$. The Me-3-epi-GA_1 fraction, which still contained impurities, was chlorinated with triphenylphosphine and carbon tetrachloride (Duri et al., 1981). The procedure was modified to take advantage of the increased reaction rate that occurs in solvents with a high dipole moment (Appel, 1975). Me-3-epi-GA_1 (378 mg) was dissolved in 10 mL of CH_2Cl_2, and then 6 mL of carbon tetrachloride, 810 mg of triphenylphosphine, and 10 mL of CH_3CN were added. After 2 h at room temperature, the solvents were removed under reduced pressure at 35 °C and the residue was separated by stepwise elution with EtOAc in hexane from a column packed with silica gel. This was followed by flash chromatography on silica gel (J. T. Baker, Inc.) using EtOAc/hexane (60:40 v/v). The fraction containing Me-3-Cl-GA_{20} was further purified by means of HPLC using a Whatman Partisil M9 10/50 preparative column eluted at 9 mL/min with 2-propanol/CH_3CN/CH_2Cl_2/hexane (16:192:192:600 v/v) and monitored at 210 nm. The chlorine was removed by hydrogenolysis with tributyltin hydride in the presence of 2,2′-azobis(isobutyronitrile) (Duri et al., 1981). The Me-GA_{20} fraction was purified by flash chromatography on silica gel using EtOAc/hexane (60:40 v/v). The Me-GA_{20} fraction was hydrolyzed (Beale et al., 1980) and GA_{20} was purified by means of a Nucleosil 5N$(CH_3)_2$ column eluted at 1 mL/min with 0.05% (v/v) HOAc/MeOH. The synthetic Me-TMS-GA_{20} had the same spectra and KRI as authentic Me-TMS-GA_{20} by GC–MS.

GC–MS. The fractions were derivatized by using ethereal diazomethane and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) (Pierce Chemical Co.), and GC–MS was performed as previously described (Poling and Maier, 1988). For the navel orange fractions, 25 μL of MSTFA was used and 2–4 μL of undiluted sample was injected. The reference samples were derivatized with 25–50 μL of MSTFA and diluted with methylene chloride as needed. A solution of Parafilm plus triacontane in hexane was co-injected with the sample to determine the KRIs (Gaskin et al., 1971).

RESULTS AND DISCUSSION

Table I shows the HPLC and GC–MS data of the putative GAs from immature navel orange fruit and the comparative data for the reference materials. The fractions in which the GAs eluted from the C_{18} column are in reasonable agreement with the published values (Koshioka et al., 1983) if allowance is made for the fact that ABA elutes 1 min later on our system. The only difference is that GA_8 might have been expected to elute earlier than the fraction containing GA_{29}. The ranges of the RRt-(GA_3)s from the Nucleosil 5N$(CH_3)_2$ column also show a reasonable overlap. In some cases the GAs were split between two fractions, e.g., GA_1, and each fraction in which the GA occurred is listed in Table I. The identification of GA_1, GA_4, GA_9, GA_{17}, GA_{19}, GA_{20}, and GA_{29} is based on the comparison of the full-scan mass spectra (data not shown) and the KRIs. In addition, the mass spectra and KRIs of GA_9 and GA_{20} from navel orange fruit also agree with those from Progress 9 peas (data not shown). Table I shows the comparison of selected characteristic masses of the GAs from navel orange fruit to those of the reference sources. No reference material was available for GA_8, so the identification, which rests on the comparison of the full spectrum to the published spectrum (Binks et al., 1969) and of the characteristic masses (Crozier and Durley, 1983; Takahashi et al., 1986), must remain tentative at this time.

GA_{29} was the most abundant GA in navel orange fruit. The amounts of the GAs were estimated from the total ion current after background subtraction. The relative amounts of GAs in orange fruit are $GA_{29} > GA_1$, GA_{19}, $GA_{20} > GA_4$, $GA_9 > GA_8$, GA_{17}. This estimate does not take into account the differences in recovery of different GAs.

In addition to the identified GAs shown in Table I, several other compounds that had GA-like mass spectra were seen in the fruit extracts. They did not appear to be any of the known GAs, but two of them had spectra similar to those of other dihydroxylated GAs. There was insufficient material to identify any of these compounds at this time.

GA_1, GA_8, GA_{17}, GA_{19}, GA_{20}, and GA_{29} are all members of the early 13-hydroxylation pathway. The pathway proceeds from GA_{44} to GA_{19} to GA_{20} and finally to GA_1, which is the only native GA in maize and pea that is required for stem elongation (MacMillan, 1987). The earlier members of the pathway have biological activity because they are metabolized to GA_1. These GAs are also found in the vegetative shoots of navel oranges (Poling and Maier, 1988). GA_4 and GA_9 are not members of this pathway. Whether or not they occur in shoots has not been determined because in the earlier study the fractions eluting from the C_{18} column after 36 min were insufficiently pure for GC–MS, even after chromatography on the Nucleosil column, and were not examined. In feeding studies with other species, GA_4 has been shown to be converted into GA_1 (Sponsel, 1983). Whether GA_9 to GA_4 to GA_1 is an alternate pathway for GA_1 biosynthesis or GA_4 and GA_9 serve a different function in the fuit remains to be determined.

These results for immature navel orange fruit are in close agreement with the reported identification of GA_1, GA_4, GA_9, GA_{19}, GA_{20}, GA_{24}, GA_{25}, GA_{44}, and GA_{53} in young fruit of Satsuma mandarin (Goto et al., 1989). GA_1, GA_3, GA_8, GA_{19}, GA_{20}, GA_{29}, 3-epi-GA_1, 2-epi-GA_{29}, and iso-GA_3 were reported in vegetative and reproductive tissues of Valencia oranges (Turnbull, 1989). This agrees with our identification of GAs of the early 13-hydroxylation pathway in navel orange fruit and shoots (Poling and

Table I. GC-MS of Putative GAs Purified from Immature Navel Orange Fruit and GAs from Reference Materials

GA	source	HPLC Rt,[a] min	RRt(GA_3)[b]	KRI[c]	constituent ions, m/z (relative intensity)			
GA_1	orange	24–27	0.49–0.85, 0.85–1.22	2687	506 (100)	491 (9)	448 (12)	447 (4)
					377 (9)	375 (9)	235 (8)	207 (18)
	synthetic			2686	506 (100)	491 (11)	448 (15)	447 (8)
					377 (10)	375 (10)	235 (6)	207 (24)
GA_4	orange	36–39	0.49–0.86	2528	418 (26)	400 (10)	386 (21)	328 (31)
					289 (67)	284 (100)	225 (79)	224 (74)
	authentic		0.69	2528	418 (24)	400 (8)	386 (19)	328 (27)
					289 (70)	284 (100)	225 (83)	224 (75)
GA_8	orange	18–24	0.46–0.85	2830	594 (100)	579 (8)	535 (8)	504 (0)
					448 (13)	379 (11)	375 (13)	238 (26)
	literature[d]				594 (100)	579 (10)	535 (10)	504 (5)
					448 (25)	379 (20)	375 (15)	238 (28)
GA_9	orange	39–56	0.87–1.24	2339	330 (7)	298 (100)	286 (16)	284 (6)
					270 (82)	243 (43)	227 (53)	226 (53)
	authentic			2337	330 (8)	298 (100)	286 (17)	284 (6)
					270 (87)	243 (46)	227 (59)	226 (56)
GA_{17}	orange	33–36	0.46–0.85	2596	492 (42)	460 (23)	433 (24)	432 (27)
					401 (13)	373 (22)	251 (16)	208 (100)
	pea		0.52–0.90	2594	492 (43)	460 (22)	433 (23)	432 (19)
					401 (14)	373 (21)	251 (21)	208 (100)
GA_{19}	orange	33–36	0.86–1.19	2620	462 (8)	447 (5)	434 (100)	402 (30)
					375 (47)	374 (58)	259 (23)	207 (34)
	pea		0.90–1.29	2619	462 (9)	447 (6)	434 (100)	402 (27)
					375 (39)	374 (45)	259 (18)	207 (28)
GA_{20}	orange	30–33	0.86–1.19, 1.19–1.52	2509	418 (100)	403 (16)	387 (2)	375 (44)
					359 (13)	301 (14)	208 (13)	207 (26)
	synthetic			2506	418 (100)	403 (16)	387 (2)	375 (47)
					359 (15)	301 (15)	208 (14)	207 (37)
GA_{29}	orange	18–24	1.20–1.56	2698	506 (100)	491 (12)	477 (4)	447 (7)
					389 (10)	375 (12)	303 (22)	207 (34)
	pea		0.90–1.29, 1.29–1.68	2696	506 (100)	491 (12)	477 (4)	447 (7)
					389 (10)	375 (14)	303 (24)	207 (44)

[a] Preparative C_{18} HPLC. [b] Analytical Nucleosil $5N(CH_3)_2$ HPLC. [c] Kovats retention index. [d] Data from Crozier and Durley (1983) and Takahashi et al. (1986).

Maier, 1988) but does not confirm the identification of GA_4 and GA_9. I found no evidence of 3-epi-GA_1 or 2-epi-GA_{29} in navel orange fruit or shoots, but this could be because of the different detection methods used. My identification of the GAs in the navel orange was based on the full-scan mass spectra, while the identification in Valencia orange was based on selected ion monitoring (except for GA_3, GA_{29}, and iso-GA_3). I also found no evidence of GA_3 or iso-GA_3 in the fruit but did identify iso-GA_3 in shoots of navel oranges. Those shoots were harvested in a commercial citrus-growing area, and it seems possible that the iso-GA_3 found in the shoots resulted from the degradation of GA_3 applied earlier in the season to improve fruit quality. The navel fruit were harvested at our laboratory, which is not located near any citrus-growing areas.

The identification of the endogenous GAs of the fruit will allow the quantitation of the individual GAs by GC-selected ion monitoring. The identification of GA_9 and GA_4 in the fruit means that in addition to measuring the amounts of GAs of the early 13-hydroxylation pathway that it may also be necessary to measure the amounts of the GAs of the non-13-hydroxylated pathway to understand GA-related responses in citrus. Deuterated GA_1 and GA_{20} are being synthesized as described by use of deuterated reagents (Duri et al., 1981). GA_4 and GA_9 can be synthesized by starting with GA_7 (Beale et al., 1980; Duri et al., 1981). This will allow the quantitation of the GAs of both pathways.

In summary, on the basis of the evidence from navel and Valencia oranges and Satsuma mandarin, it appears that the early 13-hydroxylation pathway is the major biosynthetic pathway of GAs in citrus species, although there appears to be a second pathway involving non-13-hydroxylated GAs.

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Registry No. GA_1, 545-97-1; GA_4, 468-44-0; GA_9, 427-77-0; GA_{17}, 18411-79-5; GA_{19}, 6980-44-5; GA_{20}, 19143-87-4; GA_{29}, 29774-53-6; GA_8, 7044-72-6.